REMARKS

In the Office Action dated February 23, 2005, claims 39-46, 51, 56-58, 60-68, and 86-95 are pending and are rejected.

This Response addresses each of the Examiner's rejections. Applicants therefore respectfully submit that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

Claims 39-46, 51, 56-58, 60-68 and 86-95 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement.

The rejected claims are directed to methods of inducing somatic differentiation of stem cells into neural progenitor cells (claims 39-46, 88, 95); methods of differentiating neural progenitor cells into somatic cells (claims 51, 56-58, 60-63, 86-87); and methods of producing enriched preparations of human ES cell-derived neural progenitor cells (claims 64-68, 89-94).

The Examiner contends that although the method steps as claimed are adequately described, the generation and identification of the neural progenitor cells are not adequately described. The Examiner observes that certain claims identify neural progenitor cells as expressing "at least one" of specified markers such as NCAM, nestin, vimentin or Pax-6; whereas other claims do not provide any identification step for the neural progenitor cells. The Examiner states that the markers recited in the claims are also found in cell types other than neural progenitor cells. Thus, the Examiner concludes that the identification of neural progenitor cells by only one of the markers recited in the claims, or by a unspecified combination of the recited markers, or by no markers at all, fails to adequately describe a neural progenitor cell.

Applicants respectfully submit that all independent claims (i.e., claims 39, 51, 56, 60, 64, 88-89 and 95) have been amended to define the neural progenitor cells as "capable of further differentiation to a cell selected from the group consisting of neurons, oligodendrocytes and astrocytes." Support for this amendment is found in the specification, in particular on page 22, line 29 to page 23, lines 1 and 2; page 39 to 42; and the examples on page 64 to 66 and page 76 (example 5). Independent claims 60 and 89, and dependent claim 92, additionally characterize the neural progenitor cells by certain expression markers.

Applicants respectfully submit that the specification clearly discloses the preparation of neural progenitor cells (NPCs) from human embryonic stem (hES) cells, as well as differentiation of NPCs, an intermediate cell type, to somatic cells such as neurons, oligodendrocytes and astrocytes. See, e.g., page 22, line 29 to page 23, lines 1 and 2; page 39 to 42; pages 64 to 66; and page 76. Further, the specific examples and figures of the present application provide direct evidence of possession of the claimed invention with respect to obtaining NPCs and differentiated cells such as neurons, oligodendrocytes and astrocytes. See, particularly, example 5 (beginning on page 76), and Figures 7, 9, 11-13, 15 and 24-28. Moreover, transplantation data described in the specification also demonstrate that the intermediate NPCs differentiated further to somatic cells after transplantation (see Figures 21-23, 29 and 30).

In light of the specification, the present characterization of the NPCs as capable of further differentiation into neurons, oligodendrocytes or astrocytes, and the recited method steps on how the NPCs are prepared, Applicants respectfully submit that NPCs are adequately described in full compliance with the written description requirement. Accordingly, Applicants

respectfully submit that the written description rejection under 35 U.S.C. §112, first paragraph, is overcome. Withdrawal of the rejection is therefore respectfully requested.

Claims 39-46, 51, 56-58, 60-68 and 86-95 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement.

The Examiner is of the opinion that the art indicates that directed differentiation of ES cells to generate a particular cell type of interest is unpredictable. The Examiner has referenced Verfaillie et al., Hematology 369-91 (2002), in support of her position. Furthermore, according to the Examiner, the claims are not enabled for their full scope. Specifically, the Examiner indicates that the neural progenitor cells are identified in certain claims by expression of at least one of the specified markers. However, the recited markers are also found in cell types other than neural progenitor cells. Therefore, the Examiner concludes that it would have required undue experimentation for one of skilled in the art to practice the claimed invention.

In response, Applicants respectfully submit that the Verfaillie et al. reference discusses the difficulties in attempting to derive a particular cell type from mouse embryoid bodies, which are formed by spontaneous differentiation from mouse ES cells. However, in the present case, the NPCs are derived from human ES cells under controlled differentiation conditions, for example, the use of serum-free media, which results in spheres that contain NPCs.

Further, Applicants respectfully submit that the claims have been amended to specifically delineate that the NPCs are differentiated from hES cells and that the NPCs are capable of further differentiation to a cell selected from the group consisting of neurons, oligodendrocytes and astrocytes. In certain claims, the NPCs are additionally characterized by specified expression markers.

Applicants respectfully submit that the claims, as presently recited, are supported by an enabling disclosure. In particular, the present specification clearly teaches how to prepare NPCs from hES cells and how to further differentiate the NPCs into desired somatic cells. See, e.g., page 76 (example 5). Thus, based on the present teaching, those skilled in the art would be able to generate NPCs from hES cells, and to readily determine whether an intermediate cell type generated by following the method steps according to the present invention is an NPC. Those skilled in the art could simply test the intermediate cell by culturing the cell and inducing differentiation, as disclosed in the specification. The intermediate cell would be determined to be NPC if the resultant cells are neurons, oligodendrocytes and/or astrocytes.

Therefore, Applicants respectfully submit that in light of the present teaching, those skilled in the art would be able to practice the methods, as presently claimed, without undue experimentation. Accordingly, it is respectfully submitted that the enablement rejection under 35 U.S.C. §112, first paragraph, is overcome. Withdrawal of the rejection is therefore respectfully requested.

Claims 51 and 95 are rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Thomson et al. (Science 282: 1145-1147, 1998) in view of Brustle et al. (Science 285: 754-756, 1999). Essentially, the Examiner maintains that those skilled in the art would have been motivated to apply the conditions taught by Brustle et al. in respect to mouse ES cells, to human ES cells disclosed by Thomson et al., and would have had a reasonable expectation of success. The Examiner argues that the differences between human and mouse ES cells are only in the conditions for maintaining the cells, not for differentiating the cells. As support, the Examiner has referenced Mizuseki et al. (PNAS 100: 5828-5833, 2003).

Applicants respectfully disagree with the Examiner. Specifically, Applicants respectfully submit that those skilled in the art would not have been motivated to apply the conditions taught by Brustle et al. in respect to mouse ES cells, to human ES cells disclosed by Thomson et al., in order to arrive at the claimed invention with a reasonable expectation of success.

In the first instance, Applicants respectfully submit that the conditions for maintaining the ES cells in an undifferentiated state are clearly relevant to determining the conditions for differentiating the ES cells. Further, Applicants submit that the differences between hES and mES in the culture conditions undoubtably reflect underlying differences in the gene expression profile between the two cell types. Indeed in a review article published in Biology of Reproduction 71:1772-1778, 2004 **(Exhibit A)**, Rao and Stice state that "...only between 12 and 33 of the 92 hES genes[1] were overexpressed in mES lines. The authors have attributed the limited overlaps to species differences and to potential variability in culture conditions between the different stem cell populations". See page 1775, left column, last full paragraph.

Further, it is improper for the Examiner to rely on Mizuseki et al., a post filing publication, as evidence of the requisite motivation and/or reasonable expectation of success. Nevertheless, in this particular reference, the authors provided good evidence for a striking qualitative difference between mouse and primate ES cells in neuronal differentiation under exactly the same differentiation-inducing conditions (i.e., stromal cell derived inducing activity or "SDIA"). Although both mouse and primate ES cells produced neurons, only the mouse ES cells produced cells expressing dorsal and ventral neural markers. The primate ES cells did not

[1] hES genes refer to 92 high expressing genes common among six different hES lines.

differentiate as efficiently as mouse ES cells, and produced cells expressing only dorsal neural markers. See page 5832, left column, last paragraph of Mizuseki et al.

Additional evidence showing the differences in differentiation behavior between mouse and primate (human) ES cells is reported by Xu et al., Nature Biotechnology 20:1261-1264, 2002 (**Exhibit B**). Xu et al. employed the growth factor BMP4 to derive trophoblast from hES cells, and concluded: "These results underscore fundamental differences between human and mouse ES cells, which differentiate poorly, if at all, to trophoblast." See the abstract of this reference. Xu et al. further discussed the difference between hES cells and mES cells in trophoblast formation, and suggested that a basic difference exists between mouse and human ES cells in their developmental potentials. See page 1263, left column, lines 9 to 13.

Further evidence showing the differences between mES and hES cells is reported by Xu et al., Circulation Research 91: 501-508, 2002 (**Exhibit C**). These authors concluded on page 507: "Consistent with this observation, hES cell cardiomyocyte differentiation is indeed quite different from cardiomyocyte differentiation from mES and mEC cells..... Whereas DMSO and RA enhance mEC or mES cell cardiogenesis, these compounds did not show such an effect on hES cardiomyocyte differentiation".

The foregoing clearly supports Applicants' position that those skilled in the art would not have been motivated to apply the teaching of Brustle et al. relating to mouse ES cells, to human ES cells disclosed by Thomson et al. Those skilled in the art certainly would not have had a reasonable expectation of success in combining the respective teachings of the cited references.

In fact, Thomson et al. admit that there are differences between early mouse and human development, as indicated by markers in ES and EC cells of the two species. See page

1146, column 1, lines 6-10 of Thomson et al. Hence, the Thomson reference itself suggests that citations relating to the two species should not be combined.

Moreover, the Thomson et al. reference is directed to spontaneous differentiation and does not indicate what specific cell types to which the ES cells would differentiate. If anything, the Thomson reference suggests that ES cells have the ability to form derivatives of all three embryonic germ layers and therefore could form any one of gut epithelium, cartilage, bone, smooth muscle and striated muscle, neural epithelium, embryonic ganglia and stratified squamous epithelium. There is no appreciation of any intermediate cell type, much less a NPC type. Thomson et al. also teach that hES cells can differentiate into many different cell types as part of a teratoma. However, Applicants submit that teratoma formation merely reflects the intrinsic differentiation abilities of the ES cells in the context of a unique *in vivo* environment. The observations that hES cells can move down particular lineage pathways do not suggest by any means as to how an intermediate cell type can be obtained from hES cells *in vitro*. If those skilled in the art were to follow the teaching of Thomson et al., those skilled in the art would not have focused on obtaining any intermediate cell type, as all the cells differentiated from hES cells taught by Thomson et al. were somatic cells. Therefore, those skilled in the art would not seek to combine the teaching of Thomson et al. with Brustle.

In view of the foregoing, Applicants respectfully submit that those skilled in the art would not have been motivated to combine the teaching of Brustle et al. with the teaching of Thomson et al. The Examiner has used the aid of hindsight in evaluating the present invention to support an incorrect finding of obviousness under 35 U.S.C. §103. Further, even assuming that the references were combined, Applicants respectfully submit that those skilled in the art would not have had a reasonable expectation of success, as mouse ES cells and human ES cells were

known to behave differently in differentiation. Moreover, as admitted by the examiner, the art recognizes the unpredictability in directing ES cells into differentiating towards a particular cell type. See page 9 of the Office Action.

Accordingly, it is respectfully submitted that the rejection under 35 U.S.C. §103(a) based on Thomson et al. in view of Brustle et al., is overcome. Withdrawal of the rejection is therefore respectfully requested.

As to the remaining claims, claims 56-58 and 86 are rejected under 35 U.S.C. 103(a) as unpatentable over Thomson et al. in view of Brustle et al. and Stemple et al. (Cell 71: 973-985, 1992); claims 60-63 and 87 under 35 U.S.C. 103(a) as unpatentable over Thomson, Brustle, Stemple et al. and Ben-Hur et al. (J. Neurosci. 18: 5777-5788, 1998); claims 39-46, 64-68 and 88-94 are rejected under 35 U.S.C. 103(a) as unpatentable over Thomson, Brustle and Ben-Hur.

Applicants observe that the Examiner has relied upon the combination of Thomson et al. and Brustle et al. in raising these remaining rejections. As a principal matter, Applicants reassert that those skilled in the art would not have been motivated to combine the teachings of Thomson et al. and Brustle et al.; and would not have had any expectation of success even if those skilled in the art were to combine the two references. Neither of the remaining references (Stemple et al. and Ben-Hur et al.) would cure the deficiencies in Thomson et al. or Brustle et al., or the combination thereof.

Moreover, Applicants respectfully submit that the teachings of Stemple *et al.* and Ben-Hur et al. are directed to culturing neural crest cells and cells obtained from neural tissue, respectively, and cannot be properly applied to neural progenitor cells as presently claimed, which are derived *in vitro* from human ES cells.

Accordingly, it is respectfully submitted that the rejections of claims 39-46, 56-58, 60-68 and 86-94 under 35 U.S.C.§103 based on Thomson et al. in view of Brustle et al., and further in view of either or both of Stemple et al. and Ben-Hur et al., are overcome. Withdrawal of the rejections is therefore respectfully requested.

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

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Minireview

Gene Expression Profiling of Embryonic Stem Cells Leads to Greater Understanding of Pluripotency and Early Developmental Events[1]

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ABSTRACT

Embryonic stem cells are characterized by their ability to propagate indefinitely in culture, maintaining a normal karyotype and their undifferentiated state. They have the potential of differentiating into any specialized cell type in the body. An understanding of the transcriptional profile related to pluripotency and early development is necessary to better tap their developmental potential and also maintain their undifferentiated phenotype. Currently, several techniques are in use to ascertain the gene expression profile of embryonic stem cells. This review summarizes the information generated using microarray and other approaches on the gene expression analyses of stem cells in both mouse and human cell lines. We also discuss specific approaches useful in future studies aimed at further deciphering the pluripotent nature of human embryonic stem cells.

developmental biology, early development, embryo, gene expression, gene regulation, pluripotency, stem cells

INTRODUCTION

Embryonic stem (ES) cells are undifferentiated cells that have piqued scientific curiosity primarily due to their inherent pluripotent nature. The isolation of human ES cells [1, 2] has generated enormous interest due to their ability to differentiate into derivatives of all three embryonic germ layers and form virtually any cell type in the body. Two major areas of study in current ES cell research include analyses and maintenance of pluripotency involving the continued culture of the cells in an undifferentiated state, and development of uniform and directed differentiation strategies for the production of different cell types of specific interest. Although proof of principle of human ES cells developing into many differentiated phenotypes has been demonstrated [3], successful in vitro differentiation will require a well-characterized starting pluripotent ES population.

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Transcriptional analyses of the pluripotent state of human ES cells will help uncover or further define signaling pathways and molecular mechanisms involved in the maintenance of the undifferentiated state and initial loss of pluripotency. A detailed understanding of these molecular mechanisms will thus be essential for developing human ES cells as in vitro model systems for studying embryonic development and for harnessing the differentiation potential that makes them highly attractive for cell-based therapies. The following review summarizes the methodologies used and the information gathered on the transcriptional analyses of mouse and human ES cells and discusses possible mechanisms and expression profiles that characterize the pluripotency state in these cell types.

TECHNIQUES UTILIZED TO CHARACTERIZE GENE EXPRESSION PROFILE OF STEM CELLS

Numerous techniques have been used for characterizing the gene expression profile of stem cells and this has been achieved typically in comparison with differentiated counterparts or with somatic or nonpluripotent cell types. These techniques include serial analysis of gene expression [4, 5], subtractive hybridization [6, 7], representational difference analysis [8, 9], cDNA microarray [10], and oligonucleotide microarray [11] technologies. These methodologies have been used to decipher genes that are specifically expressed in either ES cells, neural stem cells (NSCs), and/or hematopoietic stem cells (HSC). Although each technique has its own unique advantage, cDNA and oligonucleotide microarray technologies have gained precedence as they provide opportunities for comparison of global gene expression patterns between different populations. Specifically, they are proving to be a vital tool in the elucidation of the molecular mechanisms involved in the maintenance of pluripotency and initial differentiation events of human ES cells. Examples of commonly used cDNA arrays are GEArrays (SuperArray Bioscience Corporation, Frederick, MD) and the Atlas Array (BD Biosciences Clontech, Palo Alto, CA). These cDNA arrays are manufactured using cutting-edge, noncontact printing technology, with cDNAs deposited onto nylon membranes. An alternative approach involves custom-made microarray analysis using robotic systems to spot cDNAs or PCR-amplified fragments onto glass slides [12, 13] and offer the major advantage of using small reaction volumes in the hybridization procedures. A robust comparison of differential expression profiles is made possible through the use of dual-fluorescent dyes and differ-

TECHNIQUE	CELL POPULATION	REFERENCE
Serial Analysis of Gene Expression (SAGE)	mESC	[15]
	hHSC	[16]
	hESC	[17]
	hMSC	[18]
Representational Difference Analysis(RDA)	mNSC	[19]
Subtractive Hybridization	hHSC	[20]
Oligonucleotide Microarray	mESC	[21, 22]
	mESC, mHSC, mNSC	[23]
	mESC, mHSC, mNSC, hHSC	[24]
	mESC, mNSC, mRPC	[25]
	mESC, mNSC	[26]
	hESC	[27-30]
cDNA Microarray	mESC	[31, 32]
	hESC	[33, 34]
	mESC, mTSC	[35]
	rNSC	[36]
	mNSC, mESC, mHSC	[37]
	mNSC, mESC	[38]
	mHSC, mNSC	[39]
	mHSC	[40, 41]

FIG. 1. Summary of techniques used to characterize differential gene expression in specific stem cell populations. m, Mouse; h, human; r, rat; ESC, embryonic stem cell; NSC, neural stem cell; HSC, hematopoietic stem cell; MSC, mesenchymal stem cell; TSC, trophoblast stem cell; RPC, retinal progenitor cell.

entially labeled probes from separate cell populations. The oligonucleotide GeneChip array system (Affymetrix, Santa Clara, CA) has gained popularity and is now widely used in multiple fields of biomedical research for a stringent quantitative analysis of gene expression. This system is a variation of custom-made microarrays on glass slides and utilizes photolithography and solid-phase chemistry to produce arrays with densely packed oligonucleotide probes [11, 14]. Figure 1 summarizes the different techniques and approaches for characterizing specific populations of stem cells.

TRANSCRIPTIONAL PROFILING OF MOUSE EMBRYONIC STEM (mES) CELLS USING MICROARRAYS

Elucidation of mES Cell-Specific Genes Using cDNA Microarrays

Subsequent to the isolation of mES cells [42], a handful of genes expressed in mES cells, including *Pou5f1* (*Oct-4*) [43, 44], *Nanog* [45, 46], *Sox2* [47], and leukemia inhibitory factor (*LIF*) [48], have been identified as critical to the maintenance of pluripotency. An initial study by Kelly and Rizzino [31] was very specific in its microarray analysis of only mES cells with the monitoring of the expression of 588 known regulatory genes using Clontech Atlas Mouse cDNA expression arrays. Of the 588 genes, 292 were expressed in D3 mES and/or D3-differentiated cells with a comparative analysis indicating a downregulation of 18 genes (ES cell-enriched) by a factor of 2.5-fold or greater. Of specific interest among the mES cell-enriched genes were a number of transcription factors (e.g., *Oct-3, PEA-3*), growth factors (*FGF-4*), and cell-cycle regulators (*CCND1* and *E*) [31]. In a subsequent study, Tanaka and coworkers [49] used the NIA mouse 15K cDNA microarray to profile mES cells, extraembryonic-restricted trophoblast stem (TS) cells, and terminally differentiated mouse embryonic fibroblast (MEF) cells to identify expressed genes specific to each population [35]. Pairwise comparisons and further clustering revealed 124 ES-specific genes, 94 TS-specific genes, and 51 genes specific to both ES and TS cells (http://www.genome.org/cgi/content/full/12/12/1921/DC1). Key ES-cell-specific genes included transcription factors (*Oct3/4, Rex1, Zfp57, Dnmt3A*), cell cycle regulators (*Cdc10, cyclin B1*), matrix proteins (*Scamp1*), DNA replication (*Top2A*), signaling (*Fgf13*) and several uncharacterized genes that included embryonal stem cell-specific gene 1 (*Esg-1*). Specific evidence suggests that *Esg-1* exhibits gene expression patterns similar to that of *Oct3/4* with its expression restricted to pluripotent cell types [50]. Taken together, these two studies demonstrate the utility of cDNA

microarrays in deciphering differential expression of specific regulatory genes in mES cells.

Elucidation of mES Cell and Stemness Genes Using Oligonucleotide Arrays

The unique ability of stem cells to self-renew along with their pluripotent nature implies that all stem cells share a common genetic signature [23, 24]. Thus, the identification of common expressed genes should provide key insights into deciphering the mechanisms involved in the maintenance of the pluripotent state. Several studies have used Affymetrix oligonucleotide array technology applied to NSC, hematopoietic stem cells (HSC) in addition to mES cells to elucidate the list of genes that represented a stem cell molecular signature. Ivanova and coworkers [24] specifically focused on the gene expression profiles of fetal and adult HSCs and found that key HSC markers, such as *c-Kit, Tie1, Gata2* and *Tek* were expressed in many different populations of the hematopoietic hierarchy. It is important to note that 54% of the HSC-enriched genes observed were also previously identified by Phillips and coworkers [40], through a subtractive hybridization approach for HSC-specific gene products. To further characterize the common gene expression profile for diverse types of stem cells, comparison of the HSC-enriched genes were conducted with mES cells and NSCs; 283 genes were commonly expressed among all three populations with the key functional categories of transcriptional regulation, signal transduction, cell cycle regulation, RNA binding, and chromatin regulation. Examples of key genes identified as mES cell-specific include *Pou5f1, Gbx2, Fgf4, cyclin D1,* and *cyclin E1* (http://www.sciencemag.org/cgi/content/full/1073823/DC1). In an independent but similar study, Ramalho-Santos and coworkers [23], identified 216 genes found to be common among all three stem cell populations with the key functional categories of signaling, transcriptional regulation, cell-cycle regulation, DNA repair, and translational regulation. Examples of key genes that were identified as ES cell-specific include *Pou5f1, Rex1, Tdgf1/Cripto, Lefty1,* and *Fgf4* (http://www.sciencemag.org/cgi/content/full/1072530/DC1). Specific observations from these studies included an enrichment of genes involved in the JAK/STAT and TGFβ pathways with both pathways shown to be important for self-renewal and early development in both mES cells [51], and HSCs [52]. These pathways often interact, indicating that a complex integration of genetic signals involved in different pathways could contribute to the nature of stemness. A brief review comparing these two studies provided further insight into the molecular mechanisms involved in stem cells and produced lists of genes relevant to stem cell biology [53]. Briefly, a comparison of the list of stemness genes identified by both the research groups revealed only 15 common genes. This lack of similarity has been attributed to significant differences in methodology, stem cell populations, and stringency in data analyses.

Another study compared three types of stem cells (ES cells, NSCs, and retinal progenitor cells) [25] and obtained a list of 385 genes common among all three cell types (http://giscompute.gis.a-star.edu.sg/suppdata_stemness). Additionally, a comparison of enriched genes obtained in this study with the two earlier studies [23, 24] revealed 332 ES cell-enriched genes and 236 NSC-enriched genes, while only 10 genes were commonly expressed in the two stem cell populations. Subsequently, a comparative study of the two earlier HSC datasets [23, 24] using identical computational techniques revealed a far greater overlap of 605 genes [54]. The similarities and differences among different stem cell populations can be interpreted in two ways: 1) there is still a significant random variation in stemness gene expression studies and/or 2) there is a core set of stem cell genes along with functionally important genes specific for each stem cell population.

Rather than seeking a common transcriptional profile between different stem cell populations, D'Amour and Gage [26] focused on elucidating differences between ES cells and multipotent NSCs. Using a transgenic approach, they purified multipotent NSCs and pluripotent ES cell populations based on Sox2-EGFP expression and generated direct genetic comparisons between the two stem cell populations. *Sox2* was used, as it is strongly associated with the pluripotent phenotype and is known to act cooperatively at several promoters with *Oct4* [55, 56]. Additionally, *Sox2* expression is characteristic of a neural tissue [57] and is a regulatory element expressed in both ES cells and neural progenitor populations [58]. A total of 112 genes was determined to be present uniquely in ES cells and differentially expressed at the 1.4-fold change level in a comparison between ES cells and NSC populations. Key genes were classified under the categories of transcriptional regulation (*Pou5f1, Rex1, Gbx2, Stat6, Nanog*), RNA binding (*Esg1*), and growth factors (*Fgf4, Tdgf1, LeftyA*). This study used a homogenous and uniform starting Sox2-defined NSC and ES cell population, revealing striking dissimilarities in the gene expression profiles of the two populations and also helped define key genes solely expressed in ES cells.

MICROARRAY ANALYSES OF HUMAN EMBRYONIC STEM CELLS

Ever since the isolation of human ES (hES) cells [1, 2] and a subsequent federal restriction on isolation of new cell lines (http://stemcells.nih.gov/stemcell), numerous research groups have adopted microarray approaches to characterize some of the NIH-approved cell lines. In this review, the nomenclature for the hES cells mentioned are those defined by the provider included in the NIH human embryonic stem cell registry (http://stemcells.nih.gov/registry/index.asp). Most of these studies have focused on elucidating the transcriptional profile of hES cells and for understanding initial differentiation events. Further transcriptional analyses and comparative profiling are essential to identify the key molecular components and mechanisms that are critical to the maintenance of the undifferentiated state and pluripotency.

Elucidation of hES Cell-Specific Genes

Utilizing the Affymetrix oligonucleotide array technology, Sato and coworkers [28] identified potential genes that could define molecular mechanisms related to pluripotency. In these studies, based on comparison of undifferentiated H1-ES cells with their differentiated counterparts, they obtained a set of 918 genes enriched in H1-ES cells. Key observations from these studies highlighted a role for components of the FGF, TGFβ/BMP, and Wnt pathways in the maintenance of pluripotency. The most highly enriched genes included those known to be expressed in early embryogenesis, such as *Oct-3/4, LeftyA, LeftyB,* and *TDGF1.* Approximately 28% of the H1-ES-enriched genes corresponded to expressed sequence tags, indicating that a detailed analysis of these genes could reveal new factors that could contribute to the pluripotent state. A comparison of the 918 H1-ES-enriched genes with published mES datasets

[23] indicated an overlap of 227 genes with the key genes, including *Pou5f1, LeftyB,* and *TDGF1.* Detailed statistical analyses revealed that the probability of the observed overlap between the human and mouse datasets were far below the estimated probability for coincidental overlapping. This indicated that the observed overlapping genes between the human and mouse datasets were significant to the pluripotent state in both species. Several genes that are members of the TGFβ/BMP signaling pathway were among the overlapping genes. These studies were conducted using only the H1 line, and the authors note the possibility that different human ES lines could have different transcriptional profiles.

To elucidate similarities and differences between transcriptional profiles of different cell lines, we conducted a direct stringent comparison between datasets obtained from two hES (H1 and BG01 [59]) cell lines (unpublished data). Toward further characterizing the pluripotent state, we used a HepG2 cell conditioned medium (MEDII) to generate another pluripotent population using BG01 cells [29]. MEDII [60, 61], has been known to induce early differentiation in mES cells, while retaining key pluripotent marker expression. The datasets from the three populations (H1-ES, BG01-ES, and BG01-MEDII) have been generated using the Affymetrix microarray technology. As expected, there was a large list of genes (8400) common among the three pluripotent populations (Fig. 2). To identify genes enriched in the pluripotent populations, we compared the three datasets against a dataset obtained from a H1-differentiated population (unpublished data). Our findings indicated that, based on stringent criteria for selection of enriched genes, a total of 133 genes overlapped between the three pluripotent populations with the key genes, including *Pou5f1, LeftyA, Sox2,* and *Fgf2.* Although many genes were unique to each pluripotent population, they all shared similarities based on key functional ontologies that can define pluripotency including signaling pathways, cell cycle regulation, and transcriptional regulation. A recent study by Abeyta and coworkers [27] comparing gene expression profiles of HSF-1, HSF-6, and H9 lines also found that the three populations overlapped by 7385 Affymetrix gene probes. Key genes expressed at significant levels in all three cell lines included *Pou5f1, Sox2, Rex1, TDGF1,* and *LeftyA.* Their studies also showed that, in spite of each stem cell line possessing a unique stem cell signature, they were all capable of contributing to multiple cell fates. Shades of pluripotency could thus exist between different stem cell lines, which could lead to either preferential or enhanced differentiation toward particular cell fates.

Using high-quality oligonucleotide glass arrays, Bhattacharya and coworkers [30] have examined gene expression in six (GE01, GE07, GE09, BG01, BG02, TE06) hES cell lines. Results revealed that all six cell lines expressed multiple markers of the undifferentiated state and shared significant homology in gene expression (http://www.grc.nia.nih.gov/branches/lns/scbudata.htm). Key among the 92 genes expressed in all six lines were *Nanog, GTCM-1, connexin 43, Pou5f1,* and *TDGF1.* However, when these results were compared with earlier published mES results [23, 24], only between 12 and 33 of the 92 hES genes were overexpressed in mES cells. The authors have attributed the limited overlaps to species differences and to potential variability in culture conditions between the different stem cell populations.

An alternate approach toward deciphering molecular mechanisms underlying pluripotency has used a comparative analysis of the expression profiles of hES cell lines and human germ cell tumor (embryonal carcinoma-EC) lines. Using cDNA microarrays and hierarchical cluster analysis, Sperger and coworkers [33] have shown that five independent hES cell lines (H1.1, H7, H9, H13, and H14) clustered tightly together, thereby indicating major similarities. A total of 895 genes were expressed at significant levels in both hES and EC lines. Among the 25 most significant genes observed in hES cells were *Pou5f1, DNMT3B, CRABP1, SALL2,* and *GABRB3.* These studies also indicated that genes differentially expressed between hES and EC cells could constitute a list of genes that reflect an adaptation to tumor growth by suppression of differentiation. Through this adaptation, long-term survival and self-renewal mechanisms could be activated.

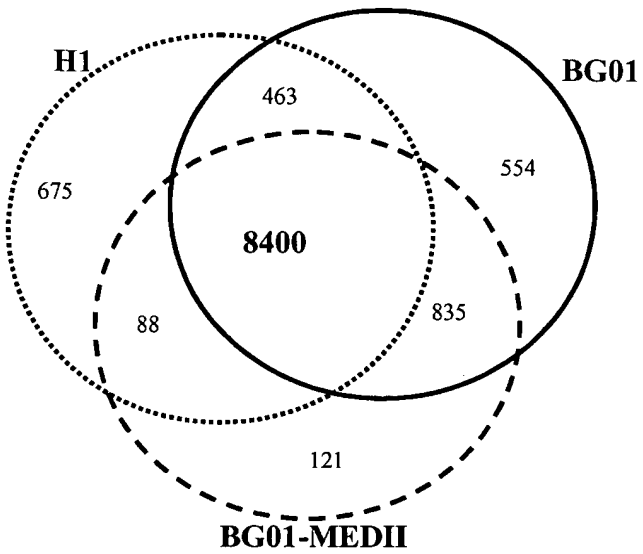

FIG. 2. Venn diagram illustrating intersection of genes from three pluripotent populations [H1(..); BG01(—); BG01-MEDII(-)] showing shared and unique gene expression. Only genes that received a present call as deciphered by the MAS 5.0 software detection algorithm were used for this comparison. Numbers are based on Affymetrix probe IDs. The region of overlap indicates the number of genes (8400) in all three populations.

Elucidation of Genes Related to Initial Differentiation Events

Microarray analyses of hES cells have primarily focused on addressing issues related to the transcriptional profiles of different stem cell lines. Most of the experimental strategies adopted have involved a comparison of undifferentiated stem cells against their differentiated counterparts. A comparison of two closely related pluripotent populations could yield specific information on key genes involved in the maintenance or exit from the pluripotent state and also aid in the development of directed differentiation strategies. We have used HepG2 conditioned medium (MEDII) [60, 61], known to induce early differentiation in mES cells, while retaining pluripotent markers, to understand early differentiation events in hES cells [29]. Microarray data using the Affymetrix system showed that MEDII treatment of BG01 hES cells retained key pluripotent marker expression (*Pou5f1, Nanog,* SSEA4), while causing regulation of genes in the TGFβ/nodal pathway. Results indicated that *TDGF1* (upregulated with MEDII treatment) and *LeftyA, Follistatin* (downregulated with MEDII treatment) were

FIG. 3. Representative genes highly expressed in embryonic stem cells. *, Italicized genes have also been observed in unpublished data involving a comparison of H1 and BG01 hES cell lines.

Transcription Factors/ Nucleic acid binding

POU5F1[23, 24, 26-31, 33, 34], GBX2[23, 24, 31], TERF1[28, 33], NANOG [28-30], HOXA11[23, 29], PITX2[23, 30], *UNG*[24, 31], PEA3[24, 31], KLF2[24, 31], KLF3/5/9[23], TTF1[24, 31], *JMJ*[28], *TOP2A*[28, 35], *AND1*[28], *SALL1*[28], *SALL2*[28, 33], *ZIC3*[28], FOXD3[27, 33], *SOX2*[26, 27, 30], ZNF43[30], PSIP1[28, 30], *RUVBL1*[23, 28], MSH2[23, 24, 27, 28], ESG1[26, 35], REX1[23, 26, 35]

Growth Factors/Receptors

FGF2[23, 24, 28], FGF4[24, 26, 31] *FGF13*[28, 35], *FGFR1/2/4*[28], BMPR1A [23, 28, 33], FZD5[28], GABABR1[23], TGFB1[23, 29], *GABABR3*[28, 33], FZD7[33], GDF3[26, 28, 30], EPHA1[33], EPHA2/B4[23, 26] , EPHA4[29]

Cell Cycle Regulators

CCNE1[23, 24, 31], *CCND1*[23, 24, 27, 28, 31], CCNB1[28, 30, 35] CDK4[23], CDC2[23, 30], *CHK2*[23, 28], *MCM3*[28],

Signaling Molecules/ Secreted Factors

TDGF1[26-30], *LEFTYA*[23, 26-30], LEFTYB[28, 30], NODAL[23, 27], PATCHED2[23, 26, 29], WNT1[23], WNT5A[29], FST[29, 30],*CRABP1*[30, 33]

Cell Adhesion/Membrane Proteins

PECAM[24, 31], LAMR1[24, 30, 31], ITGA6[23, 24, 27], BYSL[23, 24, 27], GPC4[28, 29, 33],

Miscellaneous

THY1[28], *FLJ10713*[23, 28, 33], *FLJ20105*[28], *FLJ10156*[28], *SILV*[28, 33], *DNMT3A*[35], *DNMT3B*[30, 33], *USP9X* [23, 24, 27, 28], CGI30[23, 24, 27, 28], *CGI32*[28], PPIC[23, 24, 27], LAPTM4B[23, 24, 27], KIAA1018[23, 24, 27], *KIAA0523*[28], *KIAA0922*[28], ELOVL6[23, 24, 27], ARCN1[23, 24, 27], C20ORF1[28, 30]

among the genes that were significantly differentially expressed between the two pluripotent populations. MEDII treatment of hES cells appeared to capture an event that has a gene-expression profile similar to primitive-streak stage of a developing mouse embryo.

Another early developmental event that was characterized by microarray analysis involved hES differentiation to trophoblast using BMP4 as an inducing agent [34]. Using cDNA microarrays, Xu and coworkers [34] analyzed genes differentially expressed between BMP4-treated and undifferentiated H1 hES cells. BMP4 influenced significant upregulation of key genes related to development of trophoblast and included *AP-2, MSX2, GATA2, GATA3,* and *CGβ* and a significant downregulation of genes highly expressed in pluripotent cells such as *Oct-4* and *TERT.* Of particular importance is that germ-layer (ectoderm, endoderm, and mesoderm) -specific genes were not significantly elevated in the BMP4-treated cells. These initial studies have provided opportunities for identification of potential signals that could influence initial differentiation events and mechanisms involved in the exit from the pluripotent state.

CONCLUSIONS AND FUTURE DIRECTIONS

Gene expression profiling using microarray technologies provides an important basis for revealing the molecular mechanisms involved in pluripotency and initial differentiation events that involve embryonic stem cell populations. We have summarized the research efforts that have used microarray technologies to query gene expression in stem cells, with a focus on ES cells. These studies have helped identify similarities and differences between cell lines and aided in deciphering genes that could contribute to the maintenance of the pluripotent state. Figure 3 provides a list of representative genes that contribute to the pluripotent state, compiled from the many studies summarized. The major functional categories that define pluripotency relate to transcriptional regulation, signaling, and cell cycle regulation. Specifically, genes involved in FGF, TGFβ, and Wnt signaling pathways have been implicated in early development and cell-fate decisions [62]. Recently, it has been shown that TGFβ along with LIF and FGF2 support the propagation of undifferentiated hES cells in a feeder-free culture system [63]. Further, involvement of specific

components of the Wnt signaling pathway has also been shown to maintain the undifferentiated state in both mES and hES cells [64]. These studies have provided an example for the contribution of key signaling molecules for the maintenance of the undifferentiated state of hES cells. Continued characterization and functional testing of the genes obtained from microarray studies will further our understanding of the pluripotent state.

It is important to note that utilization of microarray technologies allows potential opportunities for comparison of datasets from different groups and this facilitates comparison of different stem cell lines. However, this can be achieved only through a direct comparison of stem cell populations that share biological similarities, using uniform and stringent statistical approaches. Current bioinformatics approaches involve data integration, using ontology, to query multiple data sources, to generate relevant biological information. From a scientist's perspective, challenges remain in formally defining and representing meaningful relationships (called semantic associations) and assessing the quality of results obtained. With the potential for experimental variations between different groups and generation of disparate data from different experiments, there is usefulness for semantic-based approaches [65] to analyzing gene expression data. Briefly, semantics involves a formal description of resources so that data obtained from different experimental methodologies can be easily understood and contribute toward a unique solution. These approaches will involve a combination of data generated from different experiments and knowledge stored in ontologies, to generate specific information. There is thus a potential for explaining important biological phenomena only if they are explained in the context of the vast amounts of data from different methodologies that is being made available. Specifically, from a stem cell standpoint, there is a major scope for semantic data analysis in establishing correlations between patterns of gene expression within different pluripotent populations from one cell line or between different cell lines. In conjunction with data generated from microarray and other experimental methodologies, semantic approaches can thus aid in unraveling the mystique of the undifferentiated pluripotent state and for the development of differentiation strategies to produce cell types of interest.

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BMP4 initiates human embryonic stem cell differentiation to trophoblast

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The excitement and controversy surrounding the potential role of human embryonic stem (ES)[1,2] cells in transplantation therapy have often overshadowed their potentially more important use as a basic research tool for understanding the development and function of human tissues. Human ES cells can proliferate without a known limit and can form advanced derivatives of all three embryonic germ layers. What is less widely appreciated is that human ES cells can also form the extra-embryonic tissues that differentiate from the embryo before gastrulation. The use of human ES cells to derive early human trophoblast is particularly valuable, because it is difficult to obtain from other sources and is significantly different from mouse trophoblast. Here we show that bone morphogenetic protein 4 (BMP4), a member of the transforming growth factor-β (TGF-β) superfamily, induces the differentiation of human ES cells to trophoblast. DNA microarray, RT-PCR, and immunoassay analyses demonstrate that the differentiated cells express a range of trophoblast markers and secrete placental hormones. When plated at low density, the BMP4-treated cells form syncytia that express chorionic gonadotrophin (CG). These results underscore fundamental differences between human and mouse ES cells, which differentiate poorly, if at all, to trophoblast[3]. Human ES cells thus provide a tool for studying the differentiation and function of early human trophoblast and could provide a new understanding of some of the earliest differentiation events of human postimplantation development.

Human ES cell lines H1, H7, H9, and H14 (ref. 1) were cultured on Matrigel-coated plastic plates in conditioned medium (CM) from mouse embryonic fibroblasts and supplemented with basic fibroblast growth factor (bFGF) at 4 ng/ml to maintain their undifferentiated proliferation[4]. Recombinant human BMP4, added at concentrations of 1, 10, and 100 ng/ml to ES cells cultured in CM in the continuous presence of bFGF, induced a dose-dependent morphological change of the cells. Over a period of days, a synchronous wave of differentiation occurred, characterized by flattened, enlarged cells with reduced proliferation (Fig. 1A, B; a time-lapse film is available online (see URLs in Experimental Protocol)). The morphological changes became obvious on day 2 for BMP4 at 100 ng/ml, days 3–4 for 10 ng/ml, and days 4–5 for 1 ng/ml. BMP family members, such as BMP2 (300 ng/ml), BMP7 (300 ng/ml), and growth and differentiation factor-5 (GDF5) (30 ng/ml), induced similar morphological changes. However, other TGF-β superfamily members, such as TGF-β1 (0.01–0.1 ng/ml) and activin A (0.1–5 ng/ml), did not induce any noticeable morphological changes. The addition of inhibitors of BMP signaling, such as the soluble BMP receptor (human BMPR-IB/Fc chimera; 30 ng/ml) or the BMP-antagonizing protein noggin (300 ng/ml), blocked the morphological changes induced by the BMPs. When detached and maintained in suspension culture, the BMP4-induced cells formed vesicles (see Supplementary Fig. 1 online). ES cells cultured in unconditioned medium with or without bFGF also differentiated, but the differentiation was more asynchronous, resulting in a morphologically mixed population of cells, and this differentiation could not be blocked by the soluble BMP receptor or by noggin. BMP4 accelerated the differentiation observed in the absence of bFGF or CM. No morphological change was observed when ES cells were treated with the soluble BMP receptor or noggin alone (data not shown).

In contrast to the mononuclear cells that formed after BMP4 treatment of ES cell colonies, syncytial cells were present among individualized BMP4-treated ES cells plated at low density. For example, in one experiment in which we plated H1 cells as single cells at low density and treated them with 100 ng/ml BMP4, we observed 44 syncytia among 622 cells after two weeks of treatment. These syncytial cells contained different numbers of nuclei (from 2 to 100) and were positive for CG-β on immunostaining (Fig. 1C, D). Time-lapse movies demonstrated that these multinucleated cells formed by fusion and not by endoduplication. Injection of rhodamine-dextran confirmed that the multiple nuclei shared a continuous cytoplasm (see Supplementary Fig. 2; a time-lapse film is available online (see URLs)).

We used cDNA microarrays to analyze genes differentially expressed in the BMP4-treated and the untreated, undifferentiated H1 cells, both cultured in the continuous presence of CM and bFGF (Fig. 2A). Of 43,000 cDNA clones examined on the arrays, a cluster of only 19 clones, representing 14 genes, was strongly upregulated at all the time points examined. Of these, 11 were previously described as genes related to the development of trophoblast or placenta (Fig. 2B). Many of the genes encode transcription factors, such as transcription factor AP-2 (TFAP2)[5], msh homeobox homolog 2 (MSX2), and suppressor of cytokine signaling 3 (SSI3) (ref. 6), GATA binding proteins 2 and 3 (GATA2 and GATA3)[7], SSI3 (ref. 8), and hairy/enhancer-of-split related with YRPW motif 1 (*HEY1*)[9]. By day 7 of BMP4 treatment, there was a dramatic increase of mRNA expression of many genes expressed in trophoblast or placenta, such as those encoding CG-α and CG-β subunits, luteinizing hormone-β, and placental growth factor[10,11] (Fig. 2C). Using RT-PCR, we also observed enhanced expression of trophoblast markers, including CG-β, glial cells missing-1 (GCM1)[12], the non-classical HLA class I molecule HLA-G1, and CD9 (ref. 13) (see Supplementary Fig. 3 online). All of the top ten upregulated clones (representing eight genes) in the microarray of the day 7 BMP4-treated cells, except for one cDNA that was not studied, encode proteins or peptides previously described as being expressed by the trophoblast. These include CG-α, CG-β[10], endothelial PAS domain protein 1 (ref. 14), insulin-like growth factor–binding protein 3 (ref. 15), iodothyronine deiodinase type III (ref. 16), GATA2 (ref. 7), and glutamyl aminopeptidase[17] (see Supplementary Table 1 online). Some genes whose homologs are known to be important for mouse trophoblast, such as those encoding cytokeratin 7, human achaete scute homolog 2 (HASH2), estrogen-related receptor-β (ERR-β), and hepatocyte growth factor receptor (MET)[12], were not elevated as compared with ES cells (see Supplementary Tables 2.1 and 2.2 online). However, RT-PCR demonstrated that these genes were expressed both by the undiffer-

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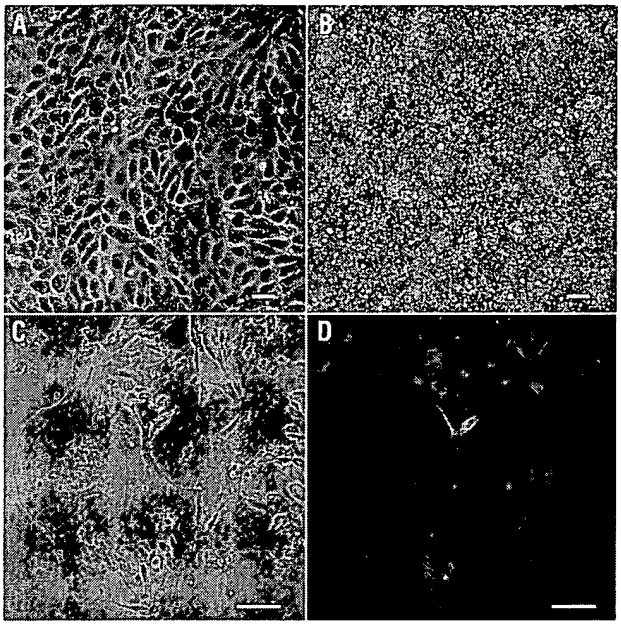

Figure 1. Morphological changes of BMP4-treated H1 cells. (A,B) H1 cells (cultured in CM with bFGF) were treated with (A) or without (B) 100 ng/ml BMP4 for seven days. (C) A syncytial cell formed after two weeks of treatment of individualized ES cells by BMP4. (D) Immunofluorescence for CG-β (green) and Hoechst 33342 fluorescence for the nuclei (blue). Bars, 25 μm.

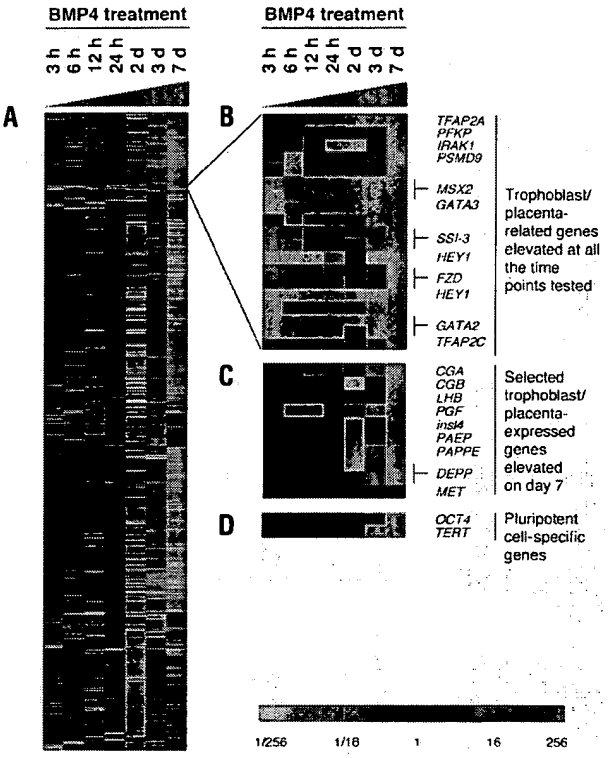

Figure 2. Microarray analysis of BMP4-treated H1 cells. H1 cells (cultured in CM with bFGF) were treated in pairs with or without 100 ng/ml BMP4. Each pair of cells was harvested at various times up to seven days. (A) Microarray of 3337 cDNA clones that showed at least threefold changes in the gene expression between a pair of BMP4-treated and untreated samples at one or more times during the treatment. Gray indicates missing or excluded data. (B–D) Expanded views of characteristic gene expression patterns. (Refer to Supplementary Tables 2.1 and 2.2 online for the raw data and gene search.)

entiated ES cells and by the BMP4-treated, differentiated cells (see Supplementary Fig. 3 online). By day 7 of BMP4 treatment, transcripts of genes highly expressed in pluripotent cells, such as those encoding POU domain, class 5, transcription factor 1 (POU5F1, also known as OCT4)[18] and telomerase reverse transcriptase (TERT)[19], were significantly decreased in both by microarray (Fig. 2D) and RT-PCR analysis (see Supplementary Fig. 3 online). Also at day 7, expression of genes characteristic of endoderm (for example, those for α-fetoprotein, hepatocyte nuclear factor, and PDX1), mesoderm (for example, those for brachyury, eomes, and chordin), and ectoderm differentiation (for example, those for cellular retinoic acid binding protein-1, sex-determining region box-2, and nestin) was not significantly elevated in the BMP4-treated cells relative to controls (see Supplementary Tables 2.1 and 2.2 online).

Because some non-trophoblast-derived tumor cell lines express trophoblast markers, we compared expression profiles of one such tumor cell line (HeLa cells[20]) with BMP4-treated human ES cells. Microarray comparison of these two kinds of cells showed that there is little similarity in their gene expression profiles. HeLa cells did express CG-α at about half the level seen in the BMP-treated ES cells (see Supplementary Fig. 4 online), but none of the other trophoblast markers that dominated the top upregulated genes in the BMP4-treated cells was expressed at high enough levels to be included in the cluster analysis for HeLa cells (a threefold ratio is required for inclusion). On the other hand, tumor markers such as the MAGE and GAGE families of tumor-associated antigens were highly expressed in HeLa cells but not in the BMP4-treated ES cells (see Supplementary Fig. 4 online).

To seek additional confirmation of trophoblast differentiation of BMP4-treated ES cells, we measured the amount of the placental hormones CG, estradiol, and progesterone secreted into the medium. H1 cells treated with BMP4 showed markedly higher concentrations of each hormone than did either undifferentiated cells or cells differentiated in unconditioned medium (Fig. 3). Fluorescence-activated cell sorting analysis of permeabilized BMP4-differentiated H1 cells labeled by an antibody to the CG-β subunit demonstrated a surprisingly uniform shift of the population to CG-β expression (Fig. 4).

To determine whether BMP4 treatment of ES cells provides an instructive signal for the differentiation of trophoblast or whether it provides a selective signal for trophoblast cells that had already committed to differentiate, we followed the fate of individual ES cells during BMP treatment by time-lapse microscopy for three days (see Supplementary Table 3 online). Over the three-day period, 119 BMP4-treated ES cells gave rise to 322 final cells, all with a flattened, differentiated morphology, and during those three days, 34 cells died and detached. In the control culture (no BMP4 treatment) during the same period, 137 ES cells gave rise to 330 cells, all with a typical ES cell morphology, and 59 cells died and detached. These results are most consistent with the model in which BMP4 has an instructive effect on trophoblast differentiation.

The first differentiation event in mammalian embryos is the formation of the trophectoderm, the outer epithelial layer of the blastocyst. The trophectoderm is crucial for implantation of the embryo and gives rise to specialized populations of trophoblast cells in the definitive placenta[12,21]. When formed into chimeras with intact preimplantation embryos, mouse ES cells rarely contribute to the trophoblast, and the manipulation of external culture condi-

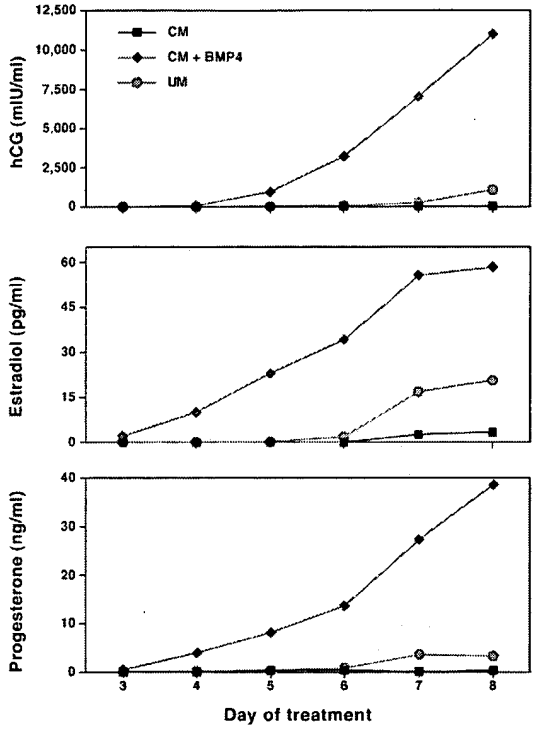

Figure 3. Immunoassays of placental hormones. Culture medium conditioned by H1 cells cultured in CM (gray square), CM + BMP4 (100 ng/ml) (red diamond), or unconditioned medium (green circle) (all in the continuous presence of bFGF) were collected at the indicated times and subjected to immunoassay for human chorionic gonadotropin (hCG), estradiol, and progesterone.

tions has, to date, failed to direct mouse ES cells to trophoblast[3]. The failure to form trophoblast is consistent with the idea that mouse ES cells are developmentally similar to primitive ectoderm, which forms after delamination of the primitive endoderm from the inner cell mass and which no longer contributes to the trophoblast[22]. However, the forced downregulation of Oct4, which is essential for ES cell pluripotency, results in a de-differentiation of mouse ES cells to trophoblast[23]. The ability of human ES cells to form trophoblasts during spontaneous, mixed differentiation in the absence of CM and bFGF[1,2] or after BMP4 induction suggests a basic difference between the developmental potential of mouse and of human ES cells. This difference is also suggested by the behavior of embryonal carcinoma cells, which are the malignant counterpart of ES cells and the stem cells of teratocarcinomas. Human teratocarcinomas often contain a trophoblast component, but mouse teratocarcinomas do not[24,25]. Mouse trophoblast stem cells have been derived from both the trophectoderm and the later extraembryonic ectoderm[26]. Mouse trophoblast stem cells can contribute to multiple trophoblast populations in chimeras and depend, in part, on fibroblast growth factor signaling for their undifferentiated propagation[26]. The human equivalent to trophoblast stem cells has not yet been derived, and it is likely that different growth factors will be required for their propagation[27]. Although, in our current studies, BMP4 efficiently induced differentiation of human ES cells to trophoblast, these trophoblast cells propagated poorly, even in the continued presence of bFGF and fibroblast feeder layers (data not shown), suggesting that additional growth factors are required for their long-term proliferation.

Human ES cells offer an important new window into early human developmental events, and the present report underlines both the power and an inherent weakness of this new model. A major strength of human ES cells is that they give access to early human cell types that were previously almost unobtainable. A major weakness is that ethical considerations will make it extremely difficult to confirm that *in vitro* results with these early cells have *in vivo* significance. We demonstrated here that BMP4 can induce human ES cell differentiation to trophoblast *in vitro;* however, a direct role of BMPs in early trophoblast differentiation *in vivo* has not, to our knowledge, been demonstrated in any mammal. Transcripts of various BMP receptors are present in morula- and blastocyst-stage mouse embryos, and transcripts of BMPs are present in the maternal tissues surrounding the embryos[28]. It has also been reported that BMP receptors are present on human ES cells[29]. The challenge for the future will be to determine whether BMP signals have a role in human trophoblast differentiation *in vivo* and to identify what signals sustain the proliferation of early trophoblast cells and direct them to become the multiple trophoblast populations of the definitive human placenta.

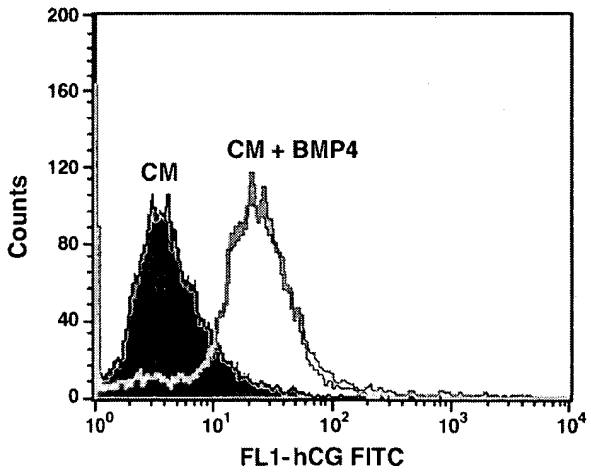

Figure 4. Fluorescence-activated cell sorting analysis for CG-β positive cells. H1 cells were cultured in CM or CM + BMP4 (100 ng/ml) for seven days. Before termination of the culture, the cells were treated with brefeldin A. The cells were individualized, fixed, and followed by fluorescence-activated cell sorting analysis with the mouse anti-human CG-β primary antibody and fluorescein-labeled rabbit anti-mouse IgG secondary antibody. hCG, human chorionic gonadotropin.

Experimental protocol

Cell culture, treatment, and syncytium analysis. Human ES cell lines H1, H7, H9, and H14 were cultured as described[1,4]. Briefly, they were plated as colonies and cultured in mouse embryonic fibroblast CM supplemented with 4 ng/ml human bFGF (Life Technologies, Rockville, MD) in six-well plates precoated with Matrigel (Becton Dickinson Labware, Bedford, MA). Protein factors were added directly to the culture in the continued presence of CM and bFGF, unless otherwise noted. Cell morphology was photographed at designated times or by time-lapse photography. For syncytium formation and analysis, H1 cells were individualized by treatment with trypsin/EDTA solution (Life Technologies) for 15 minutes at 37°C and plated at low density; some plates were then treated daily with 100 ng/ml BMP4 (R&D Systems, Minneapolis, MN, also the source for other recombinant proteins tested). Some of the individualized BMP4-treated H1 cells formed syncytial cells within two weeks of the treatment. These cells were treated with the Golgi blocker brefeldin A (Sigma, St. Louis, MO) at 1.25 μg/ml for 4 h at 37°C, fixed with 2% paraformaldehyde for 10 min, and immunos-

tained with mouse anti-human CG-β antibody (Abcam, Cambridge, UK) at 1:100 dilution and fluorescein-labeled rabbit anti-mouse IgG antibody (Pierce, Rockford, IL) at 1:200. The cells were finally stained for the nuclei with Hoechst 33342 (Sigma) and photographed under phase and epifluorescence microscopy.

DNA microarray. H1 cells were treated in pairs with or without 100 ng/ml BMP4. Each pair of the cell samples was harvested at the indicated time points during the treatment. This was followed by RNA extraction and amplification[30] and microarray analysis on DNA chips containing 43,000 cDNA clones, which represented about 30,000 unique genes[31]. Areas of the array with obvious blemishes were manually flagged and excluded from subsequent analysis. All nonflagged array elements for which the fluorescent intensity in each channel was greater than 1.5 times the local background were considered well measured. Genes for which fewer than 70% of measurements across all the samples in this study met this standard were excluded from further analysis. We selected genes for further analysis with expression concentrations that differed by at least threefold in at least one sample. The results were visualized and analyzed with TreeView software (http://rana.lbl.gov).

Immunoassays of placental hormones in the culture medium. H1 cells were treated as above. The media conditioned on the cells were collected daily from days 3–8 and tested for CG-β using the AxSYM Total hCG-β kit (Abbott, Lake Forest, IL) and for estradiol[32] and progesterone[33] concentrations.

Flow cytometry. H1 cells were cultured in CM with or without 100 ng/ml BMP4 for seven days. Before harvest, the cells were treated with brefeldin A, individualized by treatment with trypsin/EDTA solution, fixed in 2% paraformaldehyde as above, and permeabilized by suspension in PBS containing 0.1% Triton X-100. The cells were filtered through a 40-μm mesh. Then 100 μl of the cell suspension containing 5×10^5 cells per tube were added to both a test tube and a control tube; 1 μl of mouse anti-human CG-β antibody (5 mg/ml) (Abcam) was added to the test tube, and 5 μl of mouse IgG (1 mg/ml) (Sigma) were added to the control tube. The tubes were briefly vortexed to mix and incubated for 30 minutes on ice; 1 μl fluorescein-labeled rabbit anti-mouse IgG antibody (Pierce) was then added, and the tubes were incubated for another 30 minutes on ice. The cells were washed twice and finally suspended in 0.3 ml of fluorescence-activated cell sorting buffer (calcium- and magnesium-free PBS + 2% fetal bovine serum + 0.1% sodium azide) for flow cytometry. The samples were analyzed on a FACSCalibur flow cytometer (Becton Dickson, San Jose, CA) using the Cellquest acquisition and analysis software (Becton Dickson). A total of 10,000 events were acquired, and analysis was restricted to live events based on light scatter properties. The fluorescein signal was collected through a 530/30 band pass filter, and the mean fluorescences for both the IgG control (data not shown) and the test samples were determined. All data were normalized by dividing the test mean by the control mean.

URLs. For time-lapse films, see http://genome-www.stanford.edu/es-cell/index.shtml.

Note: Supplementary information is available on the Nature Biotechnology website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Characterization and Enrichment of Cardiomyocytes Derived From Human Embryonic Stem Cells

Chunhui Xu, Shailaja Police, Namitha Rao, Melissa K. Carpenter

Abstract—Cell replacement therapy is a promising approach for the treatment of cardiac diseases, but is challenged by a limited supply of appropriate cells. We have investigated whether functional cardiomyocytes can be efficiently generated from human embryonic stem (hES) cells. Cardiomyocyte differentiation was evaluated using 3 parent (H1, H7, and H9) hES cell lines and 2 clonal (H9.1 and H9.2) hES cell lines. All cell lines examined differentiated into cardiomyocytes, even after long-term culture (50 passages or ≈260 population doublings). Upon differentiation, beating cells were observed after one week in differentiation conditions, increased in numbers with time, and could retain contractility for over 70 days. The beating cells expressed markers characteristic of cardiomyocytes, such as cardiac α-myosin heavy chain, cardiac troponin I and T, atrial natriuretic factor, and cardiac transcription factors GATA-4, Nkx2.5, and MEF-2. In addition, cardiomyocyte differentiation could be enhanced by treatment of cells with 5-aza-2′-deoxycytidine but not DMSO or retinoic acid. Furthermore, the differentiated cultures could be dissociated and enriched by Percoll density centrifugation to give a population containing 70% cardiomyocytes. The enriched population was proliferative and showed appropriate expression of cardiomyocyte markers. The extended replicative capacity of hES cells and the ability to differentiate and enrich for functional human cardiomyocytes warrant further development of these cells for clinical application in heart diseases. (*Circ Res.* 2002;91:501-508.)

Key Words: human embryonic stem cells ■ cardiomyocytes ■ differentiation ■ pharmacological responses ■ cell separation

Human cardiomyocytes proliferate and mature during gestation; however, these cells terminally differentiate soon after birth.[1] It is thus generally accepted that cardiomyocytes cannot be regenerated once heart tissue is damaged by trauma such as ischemic conditions leading to cardiac infarction.[1,2] Although it appears that somatic stem cells can migrate to heart tissue and differentiate into cardiomyocytes,[3,4] such events may not be sufficient to reverse the pathological conditions. To enhance the biological function of the damaged heart, cell transplantation may be an effective therapy. Animal studies have used various types of cells for transplantation, including fetal and neonatal cardiomyocytes, skeletal and smooth muscle, fibroblasts, and bone marrow–derived cells.[4–11] Many cell types including fetal and neonatal cardiomyocytes appear to be promising candidates because of their ability to integrate into the host tissue[7,12,13] and to improve heart function.[14,15] Although this type of transplantation is promising, the source of cells such as human fetal and neonatal cardiomyocytes for cell therapies is, however, limited. This issue is particularly relevant because a significant percentage of transplanted fetal rat cardiomyocytes die posttransplantation.[16] It may therefore require either transplantation of large numbers of cardiomyocytes to achieve survival of adequate cell numbers or improvement of survival of transplanted cells.

Cardiomyocytes have been successfully derived from mouse embryonic stem (mES) cells and shown to form stable grafts in the mouse heart.[17–23] The availability of human embryonic stem (hES) cells[24,25] offers a possible solution to the poor availability of human cardiomyocytes for transplantation. hES cells have been successfully maintained in vitro for over 250 population doublings and retain stable phenotype and karyotype.[26,27] Furthermore, we have established a feeder-free system for culturing hES cells that maintains the potential of these cells to differentiate into cells of all 3 germ lineages, including beating cardiomyocytes.[27] This culture system will facilitate generation of large quantities of cells for therapeutic applications.

In the present study, we report that cardiomyocytes can be efficiently derived from hES cells using appropriate culture conditions. The cells express cardiac genes and respond appropriately to cardioactive drugs. hES cell–derived cardiomyocytes can be enriched by density separation and appear to retain appropriate phenotype, which will facilitate their use in cell replacement therapy.

Materials and Methods

Induction of Cardiomyocyte Differentiation

hES cells were maintained as described in the expanded Materials and Methods section (which can be found in the online data

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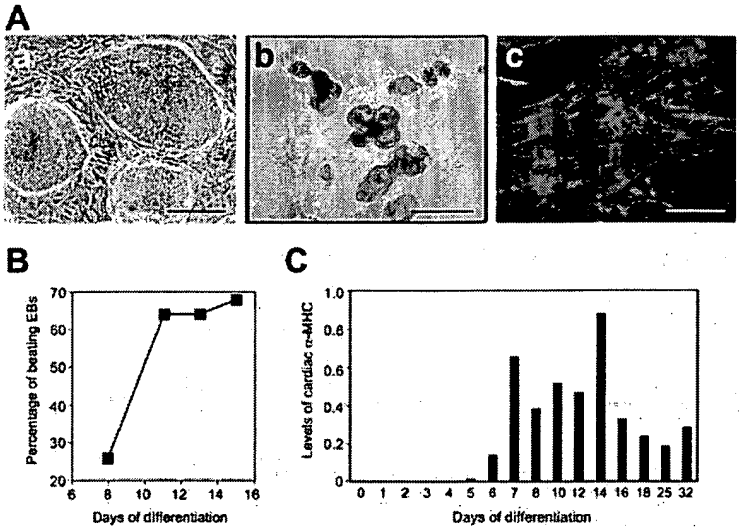

Figure 1. Differentiation of cardiomyocytes from hES cells. A, Confluent cultures of undifferentiated hES cells (a) were dissociated and cultured in suspension to form embryoid bodies (EBs) (b). EBs were transferred to gelatin-coated plates after 4 days in suspension culture to allow further differentiation into a heterogeneous cells, including spontaneously contracting cardiomyocytes that were positive for cTnI (c). Bar=400 μm for a and b and 50 μm for c. B, Percentage of EBs derived from H9.2 cells (passage 29+26, cells were subcloned from H9 at passage 29 and maintained for additional 26 passages) containing contracting cells during differentiation. C, Real-time RT-PCR analysis of cardiac α-MHC during differentiation of H1 cells (passage 29) normalized to 18S RNA.

supplement available at http://www.circresaha.org) and induced to differentiate as described below. Cells were dissociated into clumps using 200 U/mL collagenase IV (Invitrogen) at 37°C for 5 to 10 minutes and cultured in suspension using low attachment plates (Corning Inc) to form embryoid bodies (EBs). The differentiation medium contained 80% KO-DMEM, 1 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol, 1% nonessential amino acids stock, and 20% FBS (Hyclone). After 4 days in suspension, EBs were transferred onto gelatin or poly-L-lysine–coated plates at ≈1 to 3 EBs/cm^2 and cultured for additional days as described in Results. The cultures were then examined for the presence of beating cells and subjected to analysis of gene expression or pharmacological studies. The effect of the differentiation reagents dimethyl sulfoxide (DMSO), all-trans retinoic acid (RA), or 5-aza-2′-deoxycytidine (5-aza-dC), which are known to enhance cardiomyocyte differentiation in murine embryonic carcinoma (mEC) P19 cells, mES cells, or mesenchymal stem cells,[28–30] respectively, was assessed at different times during differentiation. Cultures were exposed to the reagent at the beginning of treatment and returned to basal medium without the reagent after the treatment. The number of days of differentiation includes the days in which the cells were maintained in suspension. For example, differentiation day 6 is after cells were maintained in suspension for 4 days, plated, and cultured for an additional 2 days after plating.

hES cell–derived cardiomyocytes were characterized by immunostaining and RT-PCR and evaluated in vitro for responses to pharmacological agents as described in the online data supplement.

Percoll Enrichment of Cardiomyocytes

Differentiated hES cells containing beating cells were dissociated, resuspended in differentiation medium, and loaded onto a discontinuous Percoll gradient. Percoll (Pharmacia) was diluted in a buffer containing 20 mmol/L HEPES and 150 mmol/L NaCl. The gradient consisted of a 40.5% Percoll layer over a layer of 58.5% Percoll. After centrifugation at 1500*g* for 30 minutes, cell layers were apparent. Cells at different layers were collected, washed, resuspended in the differentiation medium, and plated for immunostaining, or collected for real-time RT-PCR analysis. For immunocytochemical analysis, the fractionated cells were seeded into chamber slides, cultured for an additional few days and immunostained.

Methods for dissociation of cardiomyocytes, immunostaining and RT-PCR are provided in the online data supplement.

Results

Cardiac differentiation was initiated by inducing EB formation from undifferentiated hES cells (Figure 1A). In order to monitor the presence of beating cells in individual EBs, EBs were seeded at low density after 4 days in suspension culture, and the locations of EBs in each well were recorded. The EBs attached and continued to proliferate and differentiate into a heterogeneous population of cells including beating cardiomyocytes. Spontaneously contracting cells appeared as clusters and were identified in approximately 25% of the individual EBs at differentiation day 8 and increased to as many as 70% of the EBs by day 16 (Figure 1B). The percentage of beating EBs usually increased over time until day ≈20 and maintained at this level. In some cases, the number of beating EBs declined due to the overgrowth of other cells, which sometimes caused the peeling of cells from the plate. We found that this problem can be overcome by lowering the EB seeding density, more gently aspirating during medium exchanges, or dissociating the cells and then replating them. In our hands, contracting cells could be found in long-term cultures maintained up to differentiation day 70.

Cardiomyocyte formation in EB cultures was seen in 3 hES cell lines as well as 2 clonal lines tested (H1, H7, H9, H9.1, and H9.2). hES cells maintained for 50 passages (≈260 population doublings) retained the capacity to differentiate into cardiomyocytes (see an example in Figure 1B).

Expression of Cardiac Markers in hES-Derived Cardiomyocytes

hES cell–derived cardiomyocytes express cardiac-specific troponin I (cTnI), a subunit of the troponin complex that provides a calcium-sensitive molecular switch for the regulation of striated muscle contraction.[31] We found that cTnI was detected only in the beating regions of the culture. A representative cTnI-positive area is shown in Figure 1Ac. The presence of cTnI in the contracting cells was also confirmed by Western blot, which showed that cTnI was expressed in differentiated hES cultures containing contracting cells, but not in undifferentiated hES cells or differentiated cultures with no evidence of contracting cells (data not shown). Similar results were found in all cell lines tested.

Real-time RT-PCR assays showed that cardiac-specific α-MHC transcripts were undetectable in undifferentiated hES cell cultures or differentiated cultures at early stages, and

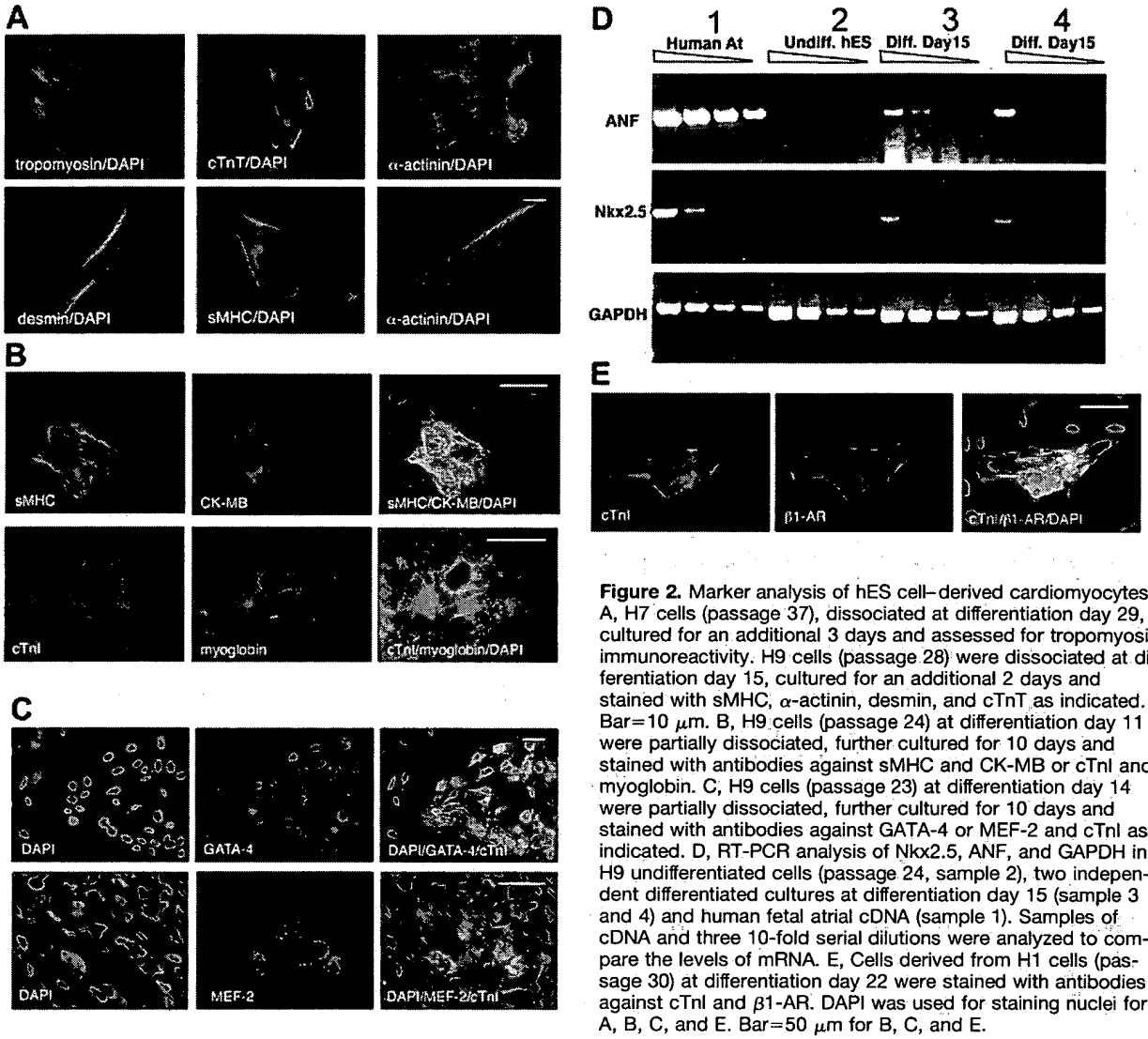

Figure 2. Marker analysis of hES cell–derived cardiomyocytes. A, H7 cells (passage 37), dissociated at differentiation day 29, cultured for an additional 3 days and assessed for tropomyosin immunoreactivity. H9 cells (passage 28) were dissociated at differentiation day 15, cultured for an additional 2 days and stained with sMHC, α-actinin, desmin, and cTnT as indicated. Bar=10 μm. B, H9 cells (passage 24) at differentiation day 11 were partially dissociated, further cultured for 10 days and stained with antibodies against sMHC and CK-MB or cTnI and myoglobin. C, H9 cells (passage 23) at differentiation day 14 were partially dissociated, further cultured for 10 days and stained with antibodies against GATA-4 or MEF-2 and cTnI as indicated. D, RT-PCR analysis of Nkx2.5, ANF, and GAPDH in H9 undifferentiated cells (passage 24, sample 2), two independent differentiated cultures at differentiation day 15 (sample 3 and 4) and human fetal atrial cDNA (sample 1). Samples of cDNA and three 10-fold serial dilutions were analyzed to compare the levels of mRNA. E, Cells derived from H1 cells (passage 30) at differentiation day 22 were stained with antibodies against cTnI and β1-AR. DAPI was used for staining nuclei for A, B, C, and E. Bar=50 μm for B, C, and E.

increased significantly after day 7 of differentiation (Figure 1C). In contrast, expression of hTERT, a gene expressed in undifferentiated hES cell cultures,[27,32] decreased during the process of differentiation (data not shown).

Other muscle markers were evaluated using dissociated hES cell–derived cardiomyocytes: sMHC, tropomyosin, α-actinin, desmin, and cardiac troponin T (cTnT) proteins were detected in single beating cells or clusters of cells (Figure 2A). Single stained cardiomyocytes showed spindle, round, and tri- or multiangular morphologies with striations characteristic of the sarcomeric structures of muscle cells. Immunostaining showed that 100% of sMHC-positive cells express cTnI, indicating that all the identified cells were cardiomyocytes. Furthermore, myogenin, a skeletal muscle–specific marker, was not detectable in the sMHC-positive cells by immunostaining, suggesting that the hES cell–derived cardiomyocytes were not expressing inappropriate proteins.

In addition to structural proteins, creatine kinase-MB (CK-MB) and myoglobin were also expressed by hES cell–derived cardiomyocytes (Figure 2B). CK-MB is found to be involved in high-energy phosphate transfer and facilitates diffusion of high-energy phosphate from mitochondria to myofibril in myocytes. Myoglobin is a cytosolic oxygen binding protein responsible for the storage and diffusion of oxygen within myocytes. Thus, hES cell–derived cardiomyocytes appear to have appropriate metabolic activity.

hES cell–derived cardiomyocytes also specifically expressed several cardiac transcription factors, including GATA-4, MEF-2, and Nkx2.5, in the differentiated cultures. These transcription factors are expressed in precardiac mesoderm and persist in the heart during development. GATA-4 immunoreactivity was found in nuclei of all cTnI-positive cells (Figure 2C). Western blots also indicated that GATA-4 is highly expressed in differentiated hES cells containing contracting cells but not in differentiated cultures that did not contain contracting cells (data not shown), indicating that GATA-4 is associated with cardiomyocyte differentiation. Similarly, MEF-2 was also expressed in nuclei of cTnI-positive cells as detected by immunostaining (Figure 2C).

Semiquantitative RT-PCR indicated that Nkx2.5 was expressed in hES cell–differentiated cultures containing beating cardiomyocytes, but undetectable in undifferentiated cultures (Figure 2D). Real-time RT-PCR analysis indicated that expression of Nkx2.5 is very low or nondetectable during H1 differentiation from day 0 to 6 and significantly increased at day 7 (data not shown). Therefore, hES cell–derived cardiomyocytes express cardiac transcription factors appropriately.

In addition, atrial natriuretic factor (ANF), a hormone that is actively expressed in both atrial and ventricular cardiomyocytes in developing heart, but is significantly downregulated in adult ventricular cells,[33] was found to be up-regulated during cardiac differentiation of hES cells as detected by a semiquantitative RT-PCR (Figure 2D).

Taken together, the above data indicate that hES cell–derived cardiomyocytes show characteristic gene expression patterns of developing cardiomyocytes.

Pharmacological Responses of hES Cell–Derived Cardiomyocytes

The in vitro function of hES cell–derived cardiomyocytes was examined by evaluating chronotropic effects of cardioactive drugs. Ion channels including L-type calcium channels play critical roles in cardiac contractile function.[34] RT-PCR analysis shows that α_1 subunit of L-type calcium channel is detected in differentiated cultures (data not shown). Therefore, we determined the effect of diltiazem, an ion channel blocker, on the beating frequency of hES cell–derived cardiomyocytes. Differentiated cells were incubated with various concentrations of the drug followed by measuring the beating frequency. Figure 3A shows that the beating frequency was decreased by diltiazem in a concentration-dependent manner; treatment with 10^{-7} mol/L diltiazem significantly reduced the frequency, and treatment with 10^{-5} mol/L stopped pulsatile contraction entirely. Contractions recovered to a normal rate 24 to 48 hours after removal of the drug. These results suggest functional ion channels exist in the hES cell–derived beating cardiomyocytes.

Cytosolic calcium is a crucial factor for controlling cardiomyocyte contraction and can be influenced by the interaction of adrenoceptors (ARs) with their ligands.[35] We therefore examined whether hES cell–derived cardiomyocytes expressed ARs by immunostaining with antibodies against AR and cTnI. The cardiomyocytes identified by cTnI expression were also immunoreactive for β_1-AR (Figure 2E) and α_1-AR (data not shown). To determine if ARs were functioning appropriately, contracting cells were treated with isoprenaline, a β_1-AR agonist, or phenylephrine, an α_1-AR agonist, and the rate of beating was monitored. As shown in Figures 3B and 3C, both isoprenaline and phenylephrine enhanced the contraction rate of hES cell–derived cardiomyocytes at differentiation day 15 to 20 in a dose-dependent manner. Unlike responses to isoprenaline or phenylephrine, cells at early stages (differentiation day 22 and 39) did not respond to clenbuterol, a β_2-AR agonist. However, cultures allowed to differentiate for a longer period of time (day 61 to 72) showed an increase in beating frequency (Figure 3E). These results suggest that differential responses of adrenoceptors occur

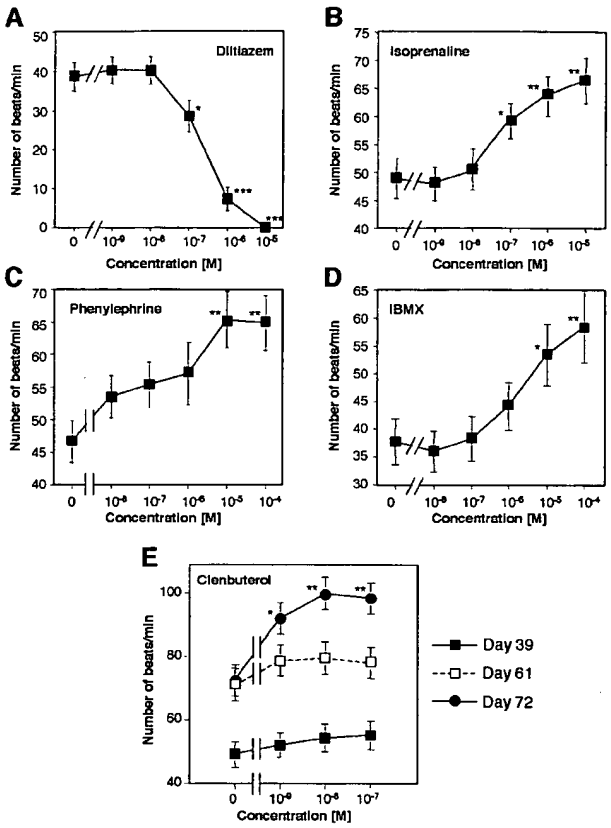

Figure 3. Studies of pharmacological responses. Effect of (A) diltiazem (a calcium channel blocker), (B) isoprenaline (a β_1-adrenoceptor agonist), (C) phenylephrine (an α1-adrenoceptor agonist), and (D) IBMX (an inhibitor of phosphodiesterases), on the contraction rate of cardiomyocytes derived from H9 cells (passage 31 to 32) or H7 cells (passage 49) at differentiation day 15 to 21. Effect of (E) clenbuterol (a β_2-adrenoceptor agonist) on H7 cells (passage 48) at differentiation day 39, 61, or 72. Each data point represents the mean±SEM pulsation rate. Statistical significance was tested by the ANOVA test: $^*P<0.05$, $^{**}P<0.005$, $^{***}P<0.0005$.

during cardiomyocyte differentiation from hES cells, similar to that seen with mES cell–derived cardiomyocytes.[36]

Application of isobutyl methylxanthine (IBMX), an inhibitor of phosphodiesterase (which converts cAMP into 5′AMP), resulted in a concentration-dependent increase of the contraction rate by IBMX (Figure 3D). These results indicate that the hES cell–derived cardiomyocytes respond appropriately to cardioactive drugs and this response may be mediated through a cAMP-dependent mechanism.[37]

Effect of Differentiation Induction Reagents on Cardiomyocyte Differentiation

In order to enhance cardiomyocyte differentiation, the effect of differentiation induction reagents was evaluated. DMSO and RA, which have been shown to induce cardiomyocyte differentiation in mEC P19 cells[28] and mES cells,[29] respectively, were evaluated but did not enhance hES cell cardiomyocyte differentiation (additional results in the online data supplement).

5-aza-dC has been shown to induce differentiation of mesenchymal stem cells presumably via demethylation of

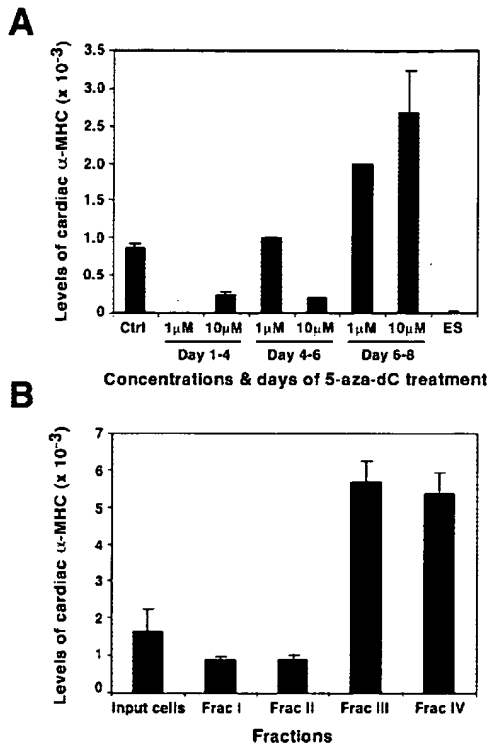

Figure 4. Enrichment of cardiomyocytes by 5-aza-dC treatment and Percoll separation. A, Effect of 5-aza-dC treatment on cardiac α-MHC mRNA levels of differentiation of H9 cells (passage 26). Cells were treated with 5-aza-dC at differentiation day 1 to 4, 4 to 6, or 6 to 8 and analyzed at differentiation day 15 for cardiac α-MHC mRNA levels by real-time RT-PCR Taqman analysis. Error bars that are not visible are smaller than the width of the symbol. ES indicates undifferentiated hES cells; Ctrl, untreated differentiated cell control. B, Effect of Percoll separation on enrichment of cardiomyocytes. H9 cells (passage 31) at differentiation day 22 were dissociated and separated by Percoll centrifugation. Cardiac α-MHC mRNA levels of cells in different fractions were compared with the starting material (input cells). 18S was used for normalization for A and B.

DNA.[30] To examine if 5-aza-dC affects cardiomyocyte differentiation of hES cells, hES cells were treated with 5-aza-dC at differentiation day 1 to 4, 4 to 6, or 6 to 8. Cells were harvested at day 15 and analyzed for cardiac α-MHC by real-time RT-PCR. Treatment of H9 or H1 cells with 5-aza-dC at day 6 to 8 significantly increased the expression of cardiac α-MHC (H9; data shown in Figure 4A). In contrast, a significant decrease in expression of cardiac α-MHC was observed when H9 or H1 cells were treated at differentiation day 1 to 4. In addition, the level of cardiac α-MHC decreased when H9 cells were treated with 10 μmol/L but not 1 μmol/L 5-aza-dC during differentiation day 4 to 6 compared with the nontreatment control. Immunostaining analysis of cTnI-positive cells indicated that the increase in α-MHC correlates with an increase in the number of cardiomyocytes (online data supplement). Therefore, 5-aza-dC appears to enhance cardiomyocyte differentiation from hES cells in a time-dependent manner. Further research is needed to characterize the complete phenotype of these cells.

Enrichment of Cardiomyocytes Using Discontinuous Percoll Gradients

In order to use hES cell–derived cardiomyocytes in therapeutic applications, it will be beneficial to produce a population of cells highly enriched for cardiomyocytes. We have used discontinuous Percoll gradients to successfully enrich hES cell–derived cardiomyocytes. An example is provided in online Table 2 (in the online data supplement available at http://www.circresaha.org) in which H7 cell–derived cardiomyocytes at differentiation day 21 were dissociated and applied to a discontinuous Percoll gradient (40.5% over 58.5%). After centrifugation, 2 layers of cells were observed: one on top of the Percoll (fraction I) and a layer of cells at the interface of the 2 layers of Percoll (fraction III). These 2 fractions, cells within the 40.5% Percoll layer (fraction II) and the 58.5% Percoll layer (fraction IV), and the starting material (input cells) were collected and cultured for 2 or 7 days before immunostaining. Although beating cells were observed in all fractions, fraction III and IV contained a higher percentage of beating cells. Quantitative analysis of triplicate wells showed that fraction III contained 36±3% sMHC-positive cells and fraction IV contained 70±5% sMHC-positive cells, whereas fraction I or II contained only 3% to 5% sMHC-positive cells 2 days after seeding (online Table 2). Compared with the starting material that contained 17±4% sMHC-positive cells, fraction IV showed a 4-fold enrichment. Similar results were observed for cells cultured for additional 7 days (online Table 2). We also applied the same separation procedure to H9 cells at differentiation day 22 and found that levels of cardiac α-MHC RNA in fractions III and IV were significantly higher than cells without the separation, confirming the enrichment (Figure 4B). Similar enrichment results (20% to 40% sMHC or cTnI-positive cells for fraction III and 50% to 70% sMHC or cTnI-positive cells for fraction IV) were observed in multiple experiments using H1 or H7 cells. These results indicate a significant enrichment of cardiomyocytes using a discontinuous Percoll gradient separation.

To characterize the Percoll-enriched cell populations, we performed immunostaining using antibodies against various markers. As shown in online Table 3, positive immunoreactivity for antibodies against cardiac α/βMHC, βMHC and sMHC was found in all cardiac cells as identified by cTnI-positive cells, but not in noncardiac cells. A representative image of cTnI and sMHC staining is shown in Figure 5. In addition, cTnI-positive cells expressed N-cadherin. Neither cardiac cells nor noncardiac cells expressed myogenin, AFP, or β-tubulin III, indicating the absence of skeletal muscle, endoderm cell types, or neurons in the Percoll-enriched culture. To examine if there were any undifferentiated hES cells in the population, surface markers for undifferentiated hES cells, SSEA-4 and Tra1-81, were analyzed. No detectable signal was found in either cardiac or noncardiac cells. Therefore, the Percoll-enriched cells did not appear to contain undifferentiated hES cells.

It has been reported that α-smooth muscle actin (SMA) is present in embryonic and fetal but not in adult cardiomyocytes.[38,39] Immunostaining results indicated that all cTnI-positive cells and a subset of cTnI-negative cells expressed

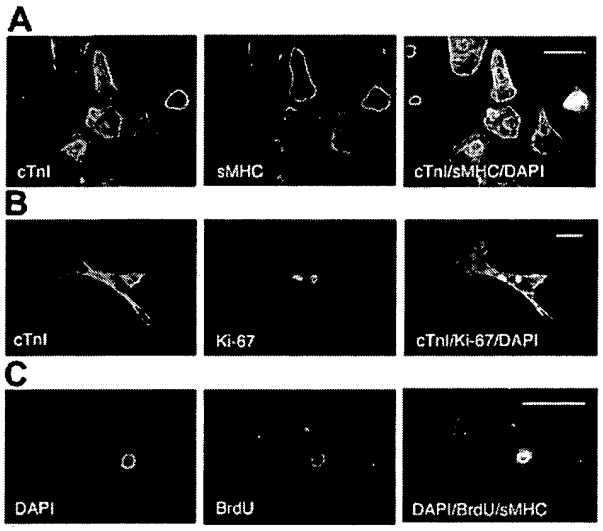

Figure 5. Characterization of Percoll-enriched cells. A, Immunostaining of H1 cells (passage 30) isolated at differentiation day 19, cultured for 2 days, and stained with antibodies against cTnI and sMHC. B, Immunostaining of H7 cells (passage 47) isolated at differentiation day 12, cultured for an additional 10 days, and stained with antibodies against cTnI and Ki-67. C, H7 cells (passage 37) isolated at differentiation day 29, cultured for additional 3 days, pulsed with BrdU, and stained with antibodies against sMHC and BrdU. Bar=50 μm

SMA, suggesting that these cardiomyocytes may represent an early stage of cardiomyocytes.

To evaluate the proliferative capacity of these cells, cultures were analyzed for BrdU incorporation and Ki-67 expression. Ki-67 is a protein in active phases of the cell cycle (G1, S, G2, and mitosis) but not in resting G0 cells and therefore used to assess cell proliferation.[40,41] In this experiment, H7 cells (passage 37) at differentiation day 13 were dissociated and isolated by Percoll separation. Cells in fraction III and IV were replated, cultured for additional 2 days, and then pulse-labeled with BrdU for 24 hours. We found that 43±4% of the sMHC-positive cells expressed BrdU, indicating that these cardiac cells were in S phase of proliferation. Parallel cultures were Percoll-separated at differentiation day 29, cultured for additional 4 days, and assessed for BrdU incorporation and the presence of Ki-67. We found that 23±10% of sMHC-positive cells incorporated BrdU and 28±4% of sMHC-positive cells were positive for Ki-67. In sMHC-negative cells, 71±2% cells incorporated BrdU and 46±7% cells were positive for Ki67. Experiments using other cultures also indicated that a subset of cTnI-positive cells expressed Ki-67 (online Table 3). Figure 5 shows a representative image. These results indicate that some of the hES cell–derived cardiomyocytes were proliferating.

Discussion

The generation of functional cardiomyocytes from hES cells has several potential applications including myocardial repair through cell transplantation. Such an application has already been demonstrated in animal models using other sources of cells[4–11]; however, the plasticity of adult stem cells has been recently challenged.[42,43] The assumed capacity of transdifferentiation of the adult stem cells into other lineages in vivo might simply be a fusing with existing cell types rather than direct conversion. In addition, adult stem cells usually have limited proliferative capacity, whereas hES cells have extended replicative capacity.[26] Therefore, hES cell–derived cardiomyocytes may prove to be the best candidate population for cell therapy. This and other potential applications of hES cell–derived cardiomyocytes are, however, largely dependent on practical aspects of producing a sufficient amount of these cells.

Our data demonstrate that hES cells can effectively differentiate into functional cardiomyocytes. This conclusion is based on (1) the contractility of the differentiated cultures, (2) specific expression of multiple cardiac-associated molecular markers by the differentiated cells, and (3) appropriate response of these differentiated cells to cardioactive drugs. While this article was in preparation, Kehat et al[44] reported that cardiomyocytes can be produced from H9.2 hES cells. In the present study, we report that cardiomyocytes can be generated from multiple hES cell lines tested (H1, H7, H9, H9.1, and H9.2) and that, using the H9.2 cells, we observed a higher percentage of beating EBs (70% versus 8%) compared with the earlier report. The difference in the efficiency of cardiomyocyte differentiation may reflect differences in culture conditions of the undifferentiated hES cells, methods used for the dissociation of hES cells to generate EBs, the length of EB suspension culture, and/or the quality of serum used for differentiation. For example, we have been maintaining undifferentiated hES cells on MEF feeders or in feeder-free conditions using medium containing serum replacement. However, Kehat et al cultured cells on feeders in medium containing FBS. Different culture conditions could lead to a different status of the hES cells used for differentiation and may be influenced by the confluence of the culture and amount of undifferentiated versus spontaneously differentiated cells in the cell population. In our experiments, cells were harvested using 200 U/mL collagenase IV for 5 to 10 minutes and gently dissociated into cell clumps for EB formation. These clumps vary in size, but the majority contained ≈100 cells or more. However, Kehat et al treated cells with 1 mg/mL collagenase IV for 20 minutes, which resulted in smaller clumps containing 3 to 20 cells. In addition, we allowed the EBs to attach onto plates after culture in suspension for only 4 days instead of 10 days as described by Kehat et al.[44] It is likely that the microenvironment within the EB culture will influence the differentiation of the cell population.

We have found that cardiomyocyte differentiation can be significantly enhanced by treatment of cells with 5-aza-dC, a demethylation reagent. This might reflect a direct improvement of cardiomyocyte differentiation due to regulation of gene expression by demethylation. Alternatively, it might simply be a net effect from the lowered efficiency of hES cell differentiation into other cell types. Our observation underscores the importance of demethylation for hES cell differentiation into cardiomyocytes and perhaps other cell types as well.

We and others have previously reported that hES cells have different properties than mES cells, including surface marker expression and response to growth factors.[24–27] Consistent with this observation, hES cell cardiomyocyte differentiation is indeed quite different from cardiomyocyte differentiation from mES and mEC cells. We observed cardiomyocyte differentiation from hES cells maintained for 260 population doublings, although cardiomyocyte differentiation using late passages of mES cells has been difficult. Whereas DMSO and RA enhance mEC P19 or mES cell cardiogenesis,[28,29] these compounds did not show such an effect on hES cell cardiomyocyte differentiation. Although the exact mechanism is unclear, it is possible that cardiomyocyte differentiation from hES cells is controlled by different signaling pathways or a common pathway that is also regulated by species-specific modulators. The effects of RA we have observed are in contrast to those reported by Schuldiner et al,[45] who showed that RA treatment increased expression of cardiac α-actin in H9.1 clonal cell line. This difference may have resulted from several factors such as different cell lines or subclones, culture systems, differentiation protocols, and/or the assay endpoints used.

In addition, we have also demonstrated the enrichment of hES cell–derived cardiomyocytes by Percoll gradient separation and proliferation capacity of the enriched cells. These cells express appropriate cardiomyocyte-associated proteins. A subset of them appears to be proliferative as determined by BrdU incorporation or expression of Ki-67, suggesting that these cardiomyocytes represent an early stage of cells. This population may be a useful model for studying cell cycle regulation of the cardiomyocytes. It will be important to determine if this represents an expandable population of cells.

In summary, we have demonstrated that an enriched population of cardiomyocytes can be derived from hES cells. These hES cell–derived cardiomyocytes can now be tested for their ability to enhance cardiac function in preclinical animal models and for utility in drug discovery.

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